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(54) Title: USE OF ANIMAL-DERIVED ANTI-MICROBIAL PEPTIDES FOR CONTROL OF PLANT PATHOGENS

(57) Abstract

The present invention is directed to the application of an animal-derived Anti-Microbial Peptide ("AMP") to the control of microbes associated with plant disease. Specifically, it has been found that the cytotoxic effects of AMP's, such as defensins and/or magainins, can be successfully used to limit the growth of bacterial and fungal pathogens associated with plant diseases.

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Description

Use of Animal-Derived Anti-Microbial Peptides for Control of Plant Pathogens

Technical Field

This invention relates to the use of animal-derived anti-microbial peptides in the control of plant-associated pathogens. More particularly, the invention relates to the use of peptides such as magainins and defensins in the control of microbes associated with plant disease.

Background of the Invention

Plant-associated microbial pathogens, such as various species of bacteria, fungi, and viruses, are responsible for significant losses in agriculture. The identification of broad spectrum agents against these microorganisms may provide enhanced strategies for plant and crop protection. Thus, the development of broad spectrum, anti-microbial agents effective against growth of plant-associated pathogens is desired.

Moreover, there is an ever-present need for new and better ways to improve agricultural production using natural agents, or compositions based upon natural agents, rather than man-made chemicals. Thus, the identification of natural agents capable of inhibitory activity against microbial plant pathogens, termed "plant-associated pathogens" herein, is desired. Particularly desired is the identification of natural agents which each display antimicrobial activity against at least two or more plant pathogen species, and especially, such agents which each display broad spectrum antimicrobial activity against different classes of plant pathogens. By the present invention, a new class of agents are provided for the control of a wide range of microbial plant-associated diseases.

It was also considered desirable to apply recombinant deoxyribonucleic acid (rDNA) and related technologies to provide for the production of such compounds in vivo to allow the modified plant to defend itself against plant-associated pathogens.

Proceeding from the seminal work of Cohen & Boyer, U.S. Patent No. 4,237,224, rDNA technology has become useful to provide novel DNA sequences and produce heterologous proteins in transformed cell cultures. In general, the joining of DNA from different organisms relies on the excision of DNA sequences using restriction endonucleases. These enzymes are used to cut donor DNA at very specific locations, resulting in gene fragments which contain the DNA sequences of interest. Alternatively, structural genes coding for desired peptides and regulatory control sequences of interest can now be produced synthetically to form such DNA Fragments.

These DNA fragments usually contain short single-stranded tails at each end, termed "sticky-ends". These sticky-ended fragments can then be ligated to complementary fragments in expression vehicles which have been prepared, e.g., by digestion with the same restriction endonucleases. Having created an expression vector which contains the structural gene of interest in proper orientation with the control elements, one can use this vector to transform host cells and express the desired gene product with the cellular machinery available. Recombinant DNA technology provides the opportunity for modifying plants to allow the expression of alien antimicrobial peptides.

However, while the general methods are easy to summarize, the construction of an expression vector containing a desired structural gene is a difficult process and the successful expression of the desired

gene product in significant amounts while retaining its biological activity is not readily predictable. Frequently, animal-derived gene products are not biologically active when expressed in plant systems.

To successfully modify plants using rDNA, one must usually modify the naturally occurring plant cell in a manner in which the cell can be used to generate a plant which retains the modification. Even in successful cases, it is often essential that the modification be subject to regulation. That is, it is desirable that the particular gene be regulated as to the differentiation of the cells and maturation of the plant tissue. In the case of anti-microbial compounds, it is also important that the modification be performed at a site where the product will be directed to contact the plant-associated pathogens of interest. Thus genetic engineering of plants with rDNA presents substantially increased degrees of difficulty.

In addition, the need to regenerate plants from the modified cells greatly extends the period of time before one can establish the utility of the genetic construct. It is also important to establish that the particular constructs will be useful in a variety of different plant species. Furthermore, one may wish to localize the expression of the particular construct in specific cell types and it is desirable that the genetically modified plant retain the modification through a number of generations.

Brief Description of the Prior Art

Magainins are reported in Zasloff, M., Proc. Nat. Acad. Sci USA 84:5449-5453 (1987) and U.S. Patent No. 4,810,777.

U.S. Patent Nos. 4,543,252, 4,659,692, and 4,705,777 are a series of related patents describing microbicidal compositions corresponding to defensins.

Disclosure of the Invention

This invention relates to the use of animal-derived anti-microbial agents in the control of plant-associated pathogens. In one aspect, the invention comprises a method of limiting growth of a plant-associated pathogen comprising contacting a plant-associated pathogen with a plant-associated pathogen-inhibiting amount of an animal-derived anti-microbial peptide under growth limiting conditions and allowing said anti-microbial peptide to act upon said plant-associated pathogen thereby limiting growth by said plant-associated pathogen.

In accordance with another aspect of the subject invention, a DNA construct is provided which comprises a transcriptional initiation region functional in plants joined at its 3' terminus to the 5' terminus of a DNA sequence which codes for at least a portion of a signal peptide which is secreted from a plant cell and an animal-derived anti-microbial peptide.

Detailed Description of the Invention

The present invention is directed to the application of an animal-derived Anti-Microbial Peptide ("AMP") to the control of microbes associated with plant disease. Specifically, it has been found that the cytotoxic effects of AMP's, such as defensins and/or magainins, can be successfully used to limit the growth of bacterial and fungal pathogens associated with plant diseases.

Plant diseases have been classified on the basis of symptoms, causative agents, specific plant organs infected, diseases involving physiological processes, diseases of specific crop groups and the physiological functions of a non-diseased plant. In this final system, diseases are grouped on the basis of their effect on one of seven essential functions: food

storage; hydrolysis and utilization of stored food; absorption of water and nutrients by roots; growth and development of meristems; water translocation in the xylem; photosynthesis; and translocation of foods through the phloem. This system focuses on the disease phenomena rather than the pathogen involved because many different kinds of pathogens can be responsible for a specific disease syndrome. Thus, a broad spectrum pathogen control agent would be valuable to treat affected plants based on the observed symptoms.

The primary agents of plant pathology are fungi, bacteria, viruses and viroids, nematodes, parasitic seed plants and a variety of non-infectious agents. As used herein, plant-associated pathogens include the fungi, bacteria, viruses and viroids which cause disease in plants but have little or no infective potential in animals.

Representative plant-associated pathogens include the following:

Fungi

More plant diseases are caused by fungi than by any other pathogenic agent. The fungi include a vast variety of living organisms which lack chlorophyll and must obtain energy from organic matter. The fungi are grouped into various classes based primarily on the morphology of their reproductive stages. The classes of fungi with plant-associated pathogens include: Plasmodiophoromycetes, Chytridiomycetes, Zygomycetes, Oomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes.

Plasmodiophoromycetes

The zoospores of Plasmodiophoromycetes penetrate root cells of the host and become ameboid, and the plasmodium develops. Only a few species in this class cause disease in cultivated plants. Plasmodiophora

brassicae, the agent of club root of crucifers, is the best known.

Chytridiomycetes

Chytrid fungi produce motile cells (zoospores) with a single flagellum. Only a few species are economically important as pathogens. A true mycelium is lacking in the species that cause plant disease. One of the best-known disease-causing chytrids is Synchytrium endobioticum, the agent of black wart of potato.

Oomycetes

The Oomycetes include many fungi causing destructive plant diseases, most of which are in one order, Peronosporales. Included are the water molds, the downy mildews, and the white blisters (white rusts). Plasmopara viticola, the cause of downy mildew of grape, and Phytophthora infestans, the cause of late blight of potato and tomato, are examples of Oomycetes. All the plant parasitic species of the class have a filamentous, branched, coenocytic mycelium that grows between or within the plant cells. The downy mildews derive their name from the abundance of mycelial branches (sporangiophores) bearing sporangia which grow out from the stomates of diseased leaves. The species of Albugo, causative agents of the white blisters, produce sporangia in compact masses under the host epidermis. A whitish blister results, and when the epidermis is ruptured a powdery white crust is evident.

Zygomycetes

Most of the Zygomycetes are saprobes, living on decaying plant and animal matter. One species, Rhizopus stolonifer, is a facultative parasite and can cause a serious rot disease of fleshy plant organs after harvest. Examples are soft rot of sweet potatoes and strawberries.

Ascomycetes

The Ascomycetes are the sac fungi, so named because the sexual or perfect-state spores are produced in a sac like cell, the ascus. The Ascomycetes are the largest class of fungal pathogens. Included are the powdery mildew fungi, which are obligate parasites.

Other examples of Ascomycetes are Claviceps purpurea, causing the ergot disease of cereals; Monilinia fructicola, the cause of brown rot of stone fruits; Venturia inaequalis, the cause of apple scab; Ceratocystis ulmi, the cause of the Dutch elm disease; and Endothia parasitica, which has nearly eliminated the American chestnut in North America.

Basidiomycetes

Examples of Basidiomycetes that cause plant disease are the smuts, rusts, and the wood-decay fungi of forest trees. Rust fungi include Puccinia graminis, the causative agent of black stem rust of cereal grains; Cronartium ribicola, the cause of white pine blister rust; and Gymnosporangium species, which cause the cedar apple and cedar hawthorn rusts. About 4000 species of rust fungi are known; all are obligate parasites.

Most of the economically important smut fungi are parasites of corn, sorghums, the cereal grains and other grasses. In the more destructive diseases, the spores develop in and destroy the flower parts so there is a total loss of grain yield. Ustilago species cause smuts of wheat, barley, corn, and oats. Species of Tilletia cause stinking smut or bunt of wheat.

The wood decay- and root rot-causing fungi of forest trees include Armillaria mellea and numerous species of Fomes and Polyporus. All of these fungi produce enormous numbers of basidiospores on gills (lamellae) or in pores or tubes on the underside of large complex fruiting bodies (basidiocarps). The

basidiocarps are referred to as mushrooms, conks, or bracket and shelf fungi.

Deuteromycetes

There are several thousand species of fungi that so far as is known reproduce only by asexual spores, called conidia. Many Deuteromycetes cause serious leaf spots and flower blights, as well as fruit rots, stem cankers, and wilts. Examples include species of Septoria, Gloeosporium, Diplodia, Alternaria, and Verticillium. The classification of the Deuteromycetes is based on the morphology of the conidial states and the mode of development of the conidia.

Bacteria

Over 100 species of bacteria mainly in five genera cause disease in hundreds of different species of flowering plants. Destructive bacterial diseases affect the major cereal, vegetable, and fruit crops. None of the bacterial pathogens of plants causes serious diseases of humans or animals.

Each species of bacterium produces a distinctive pattern of symptoms on those hosts that it attacks. The main genera of bacterial plant pathogens, and the symptoms their infections cause, include:

Agrobacterium species cause crown gall, twig gall, cane gall and hairy root diseases;

Corynebacterium species cause ring rot, tomato wilt, fruit spot and fasciation;

Erwinia species cause various forms of blight, wilt and soft rot;

Pseudomonas species cause leaf spots, olive galls, banana wilt, lilac blight, canker and bud blast;

Xanthomonas species cause leaf spots, cutting rot, black venation, bulb rot, citrus canker and walnut blight;

Streptomyces species cause potato scab and soil rot of sweet potato; and

Rhizobium species cause root nodules on legumes.

With a few exceptions, most of the bacteria that cause disease in plants are non-spore-forming, rod-shaped, gram-negative cells. Some species give a positive reaction with the Gram stain; these include species of Streptomyces and Corynebacterium, a few species of Bacillus that cause soft rots, and those species of Clostridium that can decay plant storage organs under anaerobic conditions. Only the species in the first two genera can form endospores. Rickettsia-like bacteria have been observed in diseased plants; unlike other plant pathogenic bacteria, these have been extremely difficult to grow in culture or have not been isolated.

A number of yellow-type diseases previously considered to be caused by viruses have now been shown to be caused by mycoplasma-like organisms (MPLO), members of the Mollicutes. Pleomorphic MPLOs lacking cell walls have been observed in vascular tissues of yellow-affected plants; however, some have not as yet been characterized through isolation and cultivation techniques. The MPLOs cultured from plants affected by the corn stunt and citrus stubborn disease organisms form helical motile cells, and are now classified in a new genus, Spiroplasma.

Most plant pathogenic bacteria are capable of movement in water by means of the rotation of long threadlike flagella attached to the wall. The genus Erwinia is characterized by random arrangement of flagella on the cell surface (peritrichous); in contrast the genus Xanthomonas is characterized by a single flagellum attached at one end (polar).

It is not possible to separate plant pathogenic bacteria into species on the basis of cell morphology,

or colony or staining characteristics. Therefore, biochemical and physical tests used to differentiate bacteria in general are also used in studies on plant pathogens. Helpful techniques include serological tests, DNA hybridization, sensitivity to phages, and gel electrophoresis of proteins. However, one of the most important of the tests for identification is the demonstration of pathogenicity to a specific plant.

Viruses and Viroids

Viruses and viroids are the simplest of the various causative agents of plant disease. The essential element of each of these two pathogens is an infective nucleic acid. The nucleic acid of viruses is covered by an exterior shell (coat) of protein, but that of viroids is not.

Approximately 400 plant viruses and about 10 viroids are known. The nucleic acid of most plant viruses is a single-stranded RNA (mol. wt. 1.5-4 million); a number of isometric viruses have a double-stranded RNA (mol. wt. 10-20 million). A few viruses contain double-stranded DNA (mol. wt. 4-5 million), and several containing single-stranded DNA (mol. wt. 0.8 million) have been reported. The nucleic acid of a viroid is single-stranded RNA, but its molecular weight (about 120,000) is much lower than that of viruses.

Viruses require living cells for their replication. Some viruses, such as tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), are found in many plant species; others, such as wheat streak mosaic virus, occur only in a few grasses. The replication of a plant virus appears to proceed according to the following general scheme: Introduction of the virus to a plant through a wound; release of the nucleic acid from the protein coat; association of viral RNA (or messenger RNA of DNA

viruses) with cellular ribosomes for its translation to the proteins required for virus synthesis; replication of the nucleic acid and production of coat protein; and assembly of the nucleic acid and coat protein to form complete virus particles. The replication of viroids is not clearly understood at present. Cell-to-cell spread of viruses usually occurs, and eventually the virus spreads throughout the plant. In some plants, the cells surrounding the initially infected cells die, and the virus usually does not spread further.

Plant diseases of particular interest include: diseases caused by bacterial plant-associated pathogens such as Erwinia carotovora, Pseudomonas syringae, and Xanthomonas campestris, which have been isolated from potato, tomato, and rapeseed and cotton plants, respectively; diseases caused by fungal plant-associated pathogens such as Aspergillus flavus, Fusarium oxysporum, Phytophthora infestans, Pythium ultimum, and Whetzelinia sclerotiorum, which have been isolated from raw spanish peanuts and cotton, tomato, cotton and cabbage plants, respectively. The fungus Verticillium albo-atrium has been isolated from cotton and tomato plants.

Many other microbes are also known as plant-associated pathogens, for example Aspergillis parasiticus, Alternaria brassicae, Alternaria raphani, Leptospheria maculans, Candida poropsilosis and Phoma lingam, etc. A more detailed identification and discussion of the species comprising plant-associated pathogens is contained in Agrios, G.N., Plant Pathology, 3rd ed., Academic Press, San Diego, 1988.

Fairly recently, broad spectrum antimicrobial peptides have been discovered which function as part of the host defense system of some animals against invading microorganisms: included are the families of peptides known as defensins and magainins. The

defensins and the magainins show promise in health/veterinary-related applications because of their wide range of biocidal activity against microbes associated with common animal infections.

Mammalian defensins are low molecular weight cationic peptides (usually 29-34 amino acids in length) that have been found in human, rabbit, and guinea pig polymorphonuclear leukocytes (PMN) and rabbit alveolar macrophages. Other mammals, such as bovines, also contain antimicrobial peptides which show different protein sequences and different environmental sensitivities. Defensins are disclosed generally in U.S. Patent Nos. 4,543,252 and 4,659,692.

As used herein, the term defensin is taken to mean cationic oligopeptides of 35 or less amino acids having a sequence of the formula:

$$aa_1\text{-}aa_2\text{-cys-}aa_4\text{-cys-arg-}aa_7\text{-}aa_8\text{-}aa_9\text{-cys-}aa_{11}\text{-}aa_{12}\text{-}aa_{13}\text{-glu-arg-}aa_{16}\text{-}aa_{17}\text{-gly-}aa_{19}\text{-cys-}aa_{21}\text{-}aa_{22}\text{-}aa_{23}\text{-gly-}aa_{25}\text{-}aa_{26}\text{-}aa_{27}\text{-}aa_{28}\text{-}aa_{29}\text{-cys-cys-}aa_{32}\text{-w}$$

wherein

w is as defined in U.S. Patent No. 4,659,692, and
aa_1 is val or gly;
aa_2 is val, ile, arg, ser or phe;
aa_4 is ala, val or thr;
aa_7 is arg, lys or gly;
aa_9 is leu, leu-leu, phe or ser;
aa_{11} is leu, pro, ser or gly;
aa_{12} is pro, asn, lys, phe or ser;
aa_{13} is arg, leu, ser or gly;
aa_{16} is arg, phe or ala;
aa_{17} is ala, ser or ile;
aa_{19} is phe, tyr, asp or ser;
aa_{21} is arg, lys or thr;
aa_{22} is ile or val;
aa_{23} is arg or asn;
aa_{25} is arg, ala or val;
aa_{26} is ile or arg;
aa_{27} is his, val or phe;
aa_{28} is pro, tyr or thr;
aa_{29} is leu, arg or phe;
aa_{32} is arg, ser or pro; and
w is $(arg\ or\ lys)_{0\text{-}1}$.

Particularly preferred are defensins of the following amino acid sequences:

NP1 val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg

NP2 val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg

NP3a gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg

NP3b gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg

NP4 val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg

NP5 val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-asn-gly-val-arg-his-thr-leu-cys-cys-arg

In vivo, defensins are believed to exercise cytotoxic activity against a variety of microbes in conjunction with their release from granulocytes during phagocytosis. Defensin-cidal activity has been reported in vitro against various microorganisms, including some enveloped viruses (herpes simplex, types I and II), for example. Some microbes, such as nonenveloped viruses and Bordetella bronchiseptica, are reported to show resistance to defensins; others are inhibited at varying degrees of effectiveness.

Magainins, first reported in 1987, are a class of anti-microbial peptides isolated from Xenopus skin. The two first-characterized identified magainins (M1 and M2) each have 23 amino acids but differ by two amino acid substitutions. Both have been shown to inhibit the growth of several species of bacteria and fungi. These amphibian proteins are functionally and

structurally distinct from defensins. Magainins are disclosed generally in U.S. Patent No. 4,810,777.

As used herein, the term magainin is taken to mean the class of oligopeptides of molecular weight of 2500 or less and having an amino acid sequence of the formula:

aa_1-aa_2-aa_3-aa_4-aa_5-leu-his-ser-ala-aa_{10}-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-aa_{22}-aa_{23}

wherein

aa_1 is gly_{0-1};
aa_2 is ile_{0-1};
aa_3 is gly_{0-1};
aa_4 is lys_{0-1};
aa_5 is phe_{0-1};
aa_{10} is lys or gly;
aa_{22} is asn or lys; and
aa_{23} is ser_{0-1}.

Particularly preferred are magainins of the following amino acid sequences:

M1 gly-ile-gly-lys-phe-leu-his-ser-ala-gly-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-lys-ser

M2 gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser

M2(a) ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser

M2(b) gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser

M2(c) lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser

M2(d) phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser

M2(e) leu-his-ser-ala-lys-
lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
met-asn-ser

M3 gly-ile-gly-lys-phe-leu-his-ser-ala-lys-
lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
met-asn

According to this invention, it has been found that these animal-derived AMP's show activity against a wide range of plant pathogens as well.

By animal-derived anti-microbial peptides (AMP) is meant any protein purified from animal sources, synthetically constructed, or combination thereof, functionally based upon the defensin or magainin properties.

The nomenclature used to describe AMP compounds of the present invention follows the conventional practice wherein the amino group is presented to the left and the carboxy group to the right of each amino acid residue. In the formulae representing embodiments of the present invention, the Amino- and Carboxy-terminal groups, when not specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified.

In the amino acid sequence formulae presented herein each residue is specified by the conventional practice of using three letters from the trivial name of the amino acid and wherein the L form of any amino acid having an optical isomer is intended unless otherwise expressly indicated.

In accordance with this invention, it is possible to limit the growth of such plant-associated pathogens by contact with an animal-derived antimicrobial peptide. Contact must occur under conditions in which the antimicrobial peptides retain biological activity (i.e., within particular parameters of pH, temperature, etc.). Depending upon the circumstances, slowing of

the growth of the plant pathogen may be sufficient, and in some circumstances, a slowed growth may be preferred. In other cases, complete inhibition of the pathogen's growth may be desired. Depending upon the desired effect and the specific plant pathogens of interest, different animal-derived AMP's may be useful and the concentration of such AMP modified. The means of contact is not critical to this invention and may include any means of administering, targeting, delivering, applying, directing, or facilitating a biochemical reaction between the AMP and the target microbial plant pathogen. The animal-derived AMP may be used in any form which retains antimicrobial activity. Examples of some acceptable modes are provided in United States patents 4,543,252, 4,659,692 and 4,705,777. One skilled in the art will find other variations permissible as well.

Very little is known about the toxicity of the animal-derived AMP's to the cells of higher organisms. By this invention, the effect of these animal-derived AMP's on plant tissues becomes of interest. With respect to plant tissues, tests indicate that effects of defensins and magainins on plant cell protoplasts may be dose dependent. No cell lysis is observed at concentrations of less than 0.25mg/ml of peptide for the majority of peptides tested. Depending upon the agent, lysis of tobacco cell protoplasts was noted in the range of 2.5 to 0.25 mg/ml.

In accordance with one aspect of the subject invention, DNA constructs are provided which include the structural gene sequence for expression of selected AMP's, which constructs may be introduced into a variety of plant hosts in a variety of ways and, for example, may be present as an episomal element or integrated into the host chromosome.

The structural genes for selected AMPs can be obtained for any AMP with a known amino acid sequence and/or generation of a genomic DNA library from the source for a selected AMP. The DNA sequence containing the AMP structural gene of interest may then be joined to a wide variety of other DNA sequences for introduction into an appropriate host cell. The companion sequence will depend largely upon the nature of the host, the manner of introduction of the DNA sequence into the host, and whether episomal maintenance or integration is desired. For example, the structural gene as part of a construct may be introduced into a plant cell nucleus by micropipet injection for integration by recombination into the host genome.

Alternatively, temperate viruses may be employed into which the structural gene may be introduced for introduction into a plant host. Where the structural gene has been obtained from a source having regulatory signals which are not recognized by the plant host, it may be necessary to introduce the appropriate regulatory signals for expression.

Where a virus or plasmid, e.g., tumor inducing plasmid, is employed and has been mapped, a restriction site can be chosen which is downstream from a promoter into which the structural gene may be inserted at the appropriate distance from the promoter. Where the DNA sequences do not provide an appropriate restriction site, one can digest back portions of the DNA sequence for various times with an exonuclease, such as Bal31 and insert a synthetic restriction endonuclease site. Methods for introducing viruses and plasmids into plants are described in the literature (e.g., Matzke and Schulton, J. Mol. App. Genetics 1:39-49 (1981)).

Of particular interest is the use of a tumor-inducing plasmid, e.g., Ti or Ri, where the AMP structural gene may be integrated into plant cell chromosomes. By employing the Ti-DNA right and left borders, where the borders flank an insert comprising the AMP structural gene under transcriptional and translational regulatory signals recognized by the plant host, the construct may be integrated into the plant genome and provided for expression of the AMP in the plant cell at various stages of differentiation. As transcriptional and translational regulatory regions, conveniently a signal peptide from a barley thionin may be employed which allow for transport of the AMP out of the plant cell. Promoters and/or terminators may be employed, including promoters that would provide for inducible expression or regulated expression and a plant host. Promoter regions which may be used from the Ti plasmid include the octopine synthase promoter, nopaline synthase promoter, agropine synthase promoters, or the like. Other promoters include viral promoters such as CaMV region VI promoter or full length promoter, the promoters associated with the ribulose-1,5-bisphosphate carboxylate genes, e.g., the small sub-unit genes associated with phaseolin, protein storage, cellulose formation, or the like.

The various sequences may be joined together in conventional ways. The promoter region may be identified by the region being 5' from the structural gene, for example, the opine gene, and by restriction mapping and sequencing may be selected and isolated. Similarly, the terminator region may be isolated as the region 3' from the structural gene. The sequences may be cloned and joined in the proper orientation to provide for constitutive expression of the structural gene in a plant host. By modifying crop plant cells by introduction of a functional structural gene expressing

a selected AMP, one can provide a wide variety of crops which have enhanced resistance to a broad spectrum of plant-associated pathogens, without requiring continual crop treatment and while leaving the crop relatively unaffected. In this manner, substantial economies can be achieved in labor and materials while minimizing the detrimental effects of the plant associated pathogens.

The AMP structural gene may be introduced into a wide variety of plants, both monocotyledon and dicotyledon, including maize, wheat, soy bean, tobacco, cotton, tomatoes, potatoes, Brassica species, rice, peanuts, petunia, sunflower, sugar beet, turf grass, etc. The gene may be present in cells or plant parts including callus, roots, tubers, propagules, plantlets, seed, seedlings, pollen, or the like. The AMP structural gene will manifest its activity by limiting the growth of plant associated pathogens which contact the genetically modified plant or cells thereof.

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Experimental

In the experimental disclosure which follows, all weights are given in grams (g), milligrams (mg) or micrograms (μg), all concentrations are given as percent by weight (%), molar (M), millimolar (mM) or micromolar (μM), and all volumes are given in liters (l) or milliliters (ml) unless otherwise indicated.

In order to demonstrate the use of AMP compounds in accordance with the present invention, the following examples demonstrate the inhibitory effects of AMPs applied to Plant-Associated Pathogens.

Example 1: Defensin AMP Compounds

Defensin AMP compounds may be obtained as follows: AMP compounds known as Defensins, including rabbit neutrophil proteins ("NP") NP1, NP3A, NP5 and human neutrophil proteins ("HNP") HNP1, can be prepared as described in U.S. Patent Nos. 4,543,252, 4,659,692 and 4,705,777.

One mg of each pure (verified by NMR) lyophilized defensin protein preparation to be employed is resuspended in 100μl of 0.01% (v/v) filter sterilized acetic acid, respectively.

It has been found that commercially available agar may contain contaminants which inhibit the action of defensins. For testing of defensins, a medium with low contaminants is needed. A representative example of such a medium includes Czapek Dox Broth (Difco) plus 1.5% purified agarose (Sea Plaque® agarose from FMC).

Example 2: Magainin AMP Compounds

Magainin AMP compounds may be obtained as follows: Magainins can be prepared as described in U.S. Patent No. 4,810,777. Magainins M1 and M2 can also be obtained commercially (for example, United States Biochem Corp., OH: catalog nos. 18649 and 18652, respectively).

500μg of each lyophilized Magainin protein preparation obtained as described is resuspended in 50μg sterile distilled H_2O, respectively.

Example 3: Fungal Plant-Associated Pathogens

The inhibition of fungal PAP growth by application of AMPs is demonstrated employing defensins and magainins in AMP-filled plug wells or AMP saturated paper disks (BBL/Becton Dickenson Co., MD: catalog #31039) on petri-dishes containing a medium such as Czapek Dox Broth (Difco) plus 1.5% purified agarose

(Sea Plaque® agarose from FMC). Either method appears to provide satisfactory results.

In the plug-well method, wells are punched in a concentric pattern into Czapek Dox medium-filled petri-dishes (35x10mm) using a Pasteur pipet. The plates are then inoculated in the center with a 2mm fungal plug and left until fungal growth is approximately halfway between the initial plug and the wells. At this point, 2.5-20μg of the selected AMPs are added to the wells in a small volume (2-5μl). The plates are then sealed with Parafilm® and fungal growth is monitored daily for signs of inhibition.

In a paper disk method, the plate is inoculated as above and then sterile pie-shaped paper disks are placed outside of the region of fungal growth. The AMP to be tested is then pipetted onto the paper disk. All other procedures remain the same.

Fungal PAP strains employed for testing are available from the ATCC: Aspergillus flavus ATCC 15547; Aspergillus parasiticus ATCC 26691; Fusarium oxysporum f. sp. vasinfectum ATCC 16611; Pythium ultimum ATCC 56081; and Verticillium albo-atrium ATCC 18698 (isolated from V-81, tomato) and ATCC 18699 (isolated from V-43, cotton).

Fusarium oxysporum, Pythium ultimum, and both strains of Verticillium tested show significant inhibition from all magainins and defensins tested. The fungal growth was markedly limited in areas adjacent to the animal-derived AMP proteins to varying degrees. A summary of selected results of such inhibition is found in Table 1.

Table 1

Fungal Growth Inhibition Activity of AMP Compounds

Pathogen	AMP Compound						
	NP1	NP3A	NP5	HNP1	M1	M2	C
Fusarium oxysporum	+++	+++	+	+++	+	+	-
Pythium ultimum	+++	+++	+	+++	+	+	-
Verticillium albo-atrium V-81 (18698)	+++	+++	+	+++	+++	+	-
Verticillium albo-atrium V-43 (18699)	+++	++	+	+++	+	-	-

"C" is control; "+" indicates some inhibition activity; "+++" indicates relatively strong inhibition activity; "-" indicates a lack of apparent inhibition activity.

A line of precipate was noted to form in the zone of inhibition of Verticillium V43 and V81 strains with NP1, NP3A, and HNP1. The effect of the animal-derived AMP's on Aspergillus was inconclusive.

Example 4: Bacterial Plant-Associated Pathogens

Bacterial PAPs to be tested are grown in 10ml of 2YT liquid medium (16g tryptone, 10g yeast extract, 5g NaCl and 1000ml DDH_2O, pH adjusted to 7.0 with 1M NaOH) until mid-log phase (OD_{600}=approximately 0.5), washed two times with 10ml of 10mM potassium phosphate buffer, pH 7.4 and centrifuged at 2000xg for 10 minutes.

The bacteria are resuspended in buffer and the OD measured and adjusted to 0.5 (0.5 $OD_{600}=4x10^8$ cells/ml). The cells are then diluted to approximately 10^5 cells/ml in either 10mM phosphate buffer alone (control) or buffer plus AMP at final concentrations from 1-10μg/ml. The treated cells are then incubated at 37°C for 1 hour. The incubation is terminated by further dilution (1/100 and 1/1000) and 100μl of the diluted cells are plated on semi-solid 2YT medium (2YT media plus 1.5% agar) in duplicate. The plates are then incubated overnight at 37°C and colony counts are determined.

Inhibition of bacteria is measured as the reduction in the number of colonies when compared to controls. Bacteria known to be inhibited by the magainin and/or defensin proteins, such as E. coli, S. aureus, or P. aeurginosa, are tested as controls in these examples.

Bacterial PAP strains employed for testing herein are available from the ATCC: Erwinia carotovora subsp. carotovora ATCC 25272; Pseudomonas syringae pv. tomato ATCC 10862 (U.S. Patent 3,066,080); and Xanthamonas campestris pumalvacearum XM 13 cotton ATCC9924.

At AMP concentrations of 10μg/ml, varying degrees of inhibition were seen. Complete inhibition of growth was demonstrated in some instances. A summary of selected results in found in Table 2.

Table 2

Bacterial Growth Inhibition Activity of AMP Compounds
Percent Inhibition at 10μg/ml Peptide Concentration

Pathogen	AMP Compound						
	NP1	NP3A	NP5	HNP1	M1	M2	C
Erwinia carotovora	100	69	62	59	62	99	0
Pseudomonas syringae	100	100	70	0	52	100	0
Xanthamonas campestris	100	100	34	14	92	99	0

"C" is control.

Some bacterial growth inhibition was also observed with AMPs at peptide concentrations of 1μg/ml: NP1 demonstrated strong inhibition of Erwinia (97%) and a slightly lesser inhibition of Xanthamonas (88%). Relatively strong inhibitory effects were demonstrated by NP3A for the same strains, in the range of 60-75% inhibition. Some inhibitory effects are seen in Pseudomonas as well: NP1 (22% inhibition), HNP1 (33% inhibition) and M1 (39% inhibition).

In order to demonstrate additional embodiments of the present invention, the following examples illustrate the construction of DNA sequences for expression and transport of AMPs in plant tissue and the transformation of plant tissue therewith.

Example 5: Synthesis of the Thionin Signal Peptide/NP1 DNA Sequence

The DNA sequence for expression and transport of the defensin NP1 (rabbit) is assembled from synthetic oligonucleotides using the Klenow fragment of DNA polymerase and the polymerase chain reaction (PCR) technique, as described in U.S. Patent Nos. 4,683,195 and 4,683,202.

The DNA sequence for expression and transport of NP1 consists of six non-specific base pairs followed by a BamHI site, then the DNA sequence encoding the putative signal peptide from a barley leaf-specific thionin (to allow for transport out of the cell) fused in-frame to the mature rabbit NP1 coding region, an Asp718 site, and six more non-specific base pairs. The BamHI and Asp718 sites allow the gene to be cloned into the double 35S expression cassette pCGN1431 (described below); the non-specific bases on the ends are employed to provide double-stranded helix structure on both sides of the restriction sites, a feature which most restriction enzymes require for activity. The codons

are selected in the design of this DNA sequence based on codon preferences for dicotyledonous plants (Murray et al., Nucl. Acids Res. 17:477-498 (1989)) to allow maximal translation rate in the target plants of the present example (Brassica sp.). Codon selection for other target plant species will be made as a matter of ordinary skill.

The DNA sequence which encodes the putative signal peptide of barley leaf thionin is 127bp in length. Two overlapping oligonucleotides were designed which, when annealed and extended with Klenow fragment, comprise the entire sequence: "SIG-T" is 73 "sense" strand bases, consisting of bases of 1-73 of the DNA sequence encoding the thionin signal peptide (as described by Gausing, Planta 171:241-246 (1987)):

"SIG-T":

5'-CATATCTCATTCTACTTGAGAAATTAAGGCCAACC

AGCCAACCATGGCAACCAACAAGTCTATTAAGTCCGTG-3'

"SIG-B" is 74 "anti-sense" strand bases, consisting of bases 127-54 of the DNA sequence encoding the signal peptide (Gausing, (1987) supra).

"SIG-B":

5'-GGCTTCAACTTGGACCTGCTCAAGAACCAAACCCAA

TATAAGAACACAAATAACCACGGACTTAATAGACTTGT-3'

Bases 54-73 of "SIG-T" and "SIG-B" overlap as complementary strands, and when allowed to anneal they can be extended with Klenow fragment to yield a full length double-stranded DNA fragment of 127bp.

In practice, only a relatively small fraction of the polymerase reaction products will comprise the full 127 base pairs, since the synthesis of long oligomers (>40 bases) is not highly efficient. By employing the

PCR as described below, the full length molecules can be selectively amplified, reducing the quantity of short fragments to an insignificant level.

The NP1 coding region is 102bp in length. It was constructed in a manner similar to the signal peptide DNA sequence, with "NP1-T" being "sense" bases 1-60, and "NP1-B" being "antisense" bases 102-41, based on the NP1 DNA sequence (as reported by Selsted and Harwig, Infect. Immun. 55(9):2281-2286 (1987)):

"NP1-T":

5'-GTTGTTTGCGCTTGTAGGAGAGCTTTGTGTCTTCCAAG AGAAAGGAGAGCTGGATTTTGC-3'

"NP1-B":

5'-TTATCTCCTACAGCACAATGGATGAATTCTTCCTCTA ATCCTGCAAAATCCAGCTCTCCTTT-3'

These oligonucleotides were annealed and repaired with Klenow fragment as described above.

Four smaller oligonucleotides were synthesized in order to allow fusion of the signal sequence and the NP1 sequence, facilitate addition of the restriction sites, and allow for amplification of full-length fragments from the above polymerase reactions:

"SIG-L" is a 33 base oligonucleotide consisting of six nonspecific bases, six bases comprising the BamHI site, and the first 21 "sense" bases (1-21) of the signal peptide DNA sequence:

5'-CGAGTTGGATCCCATATCTCATTCTACTTGAGA-3'

"NP1-R" is a 33 base oligonucleotide consisting of six nonspecific bases, six bases comprising the Asp718 site, and the last 21 "anti-sense" bases (102-82) of the NP1 sequence:

5'-TGAGTTGGTACCTTATCTCCTACAGCACAATGG-3'

"F-SN-T" is a 42 base oligonucleotide consisting of the last 21 "sense" bases of the signal peptide (107-127) and the first 21 "sense" bases of the NP1 sequence (1-21) :

5'-GAGCAGGTCCAAGTTGAAGCCGTTGTTTGCGCTTGTAGGAGA-3'

"F-SN-B" is a 42 base pair oligonucleotide complementary to "F-SN-T", consisting of "anti-sense" bases 21-1 of NP1 and "anti-sense" bases 127-107 of the signal peptide DNA sequence:

5 '-TCTCCTACAAGCGCAAACAACGGCTTCAACTTGGACCTGCTC-3'

When "SIG-L" and "F-SN-B" are used as primers in the PCR with the repaired signal peptide DNA fragment as template, the resulting product is a 160bp fragment with the six non-specific base pairs and BamHI site, the signal peptide DNA sequence, and 21 base pairs of the NP1 DNA sequence. The PCR is performed using a DNA Thermal Cycler machine (Perkin Elmer Cetus; Emeryville, CA) in accordance with the instructions of the manufacturer.

When "NP1-R" and "F-SN-T" are used as primers in the PCR with the repaired NP1 DNA fragment as template, the resulting product is a 135bp fragment with 21 base pairs of the signal peptide DNA sequence, the NP1 DNA sequence, the Asp718 site, and six nonspecific base pairs. Each of these reactions selectively amplifies only the full-length or near-full-length fragments from the initial repair reactions, permitting the use of crude oligonucleotides in all steps, and resulting in a great savings of time and manpower.

These two double-stranded products share a 42bp overlap which defines the fusion junction for the thionin signal sequence and the NP1 sequence. Upon strand melting (such as occurs during the PCR), a portion of each type will reanneal with an overlapping strand of the other, forming a complex which can repair into the full-length DNA sequence. When the two fragments are mixed in a 1:1 ratio, and amplified in the PCR using "SIG-L" and "NP1-R" as primers, the resulting product is the full-length sequence with the appropriate restriction sites attached.

The actual assembly of the DNA signal/NP1 sequence was carried out in several steps. One nmol each of "NP1-B" and "NP1-T" (for the NP1 region; "SIG-T" and "SIG-B" were used for the signal region) were combined with 1μl 100XTE (1XTE: 10mM Tris-HCl, 1mM EDTA) in a total volume of 100μl. This mixture is placed in a DNA Thermal Cycler (Perkin Elmer Cetus; Emeryville, CA) and annealed for 30 minutes in a cooling gradient from 80°C to 37°C followed by 15 minutes at 37°C. Ten μl of this reaction was treated with Klenow fragment in a 50μl reaction volume to repair the strands. One μl of the Klenow fragment reaction was used in a PCR reaction with "F-SN-T" and "NP1-R" ("F-SN-B" and "SIG-L") as primers to add the fusion region. The PCR conditions were as follows: 94°C for 1 min., 55°C for 2 min., 72°C for 3 min. for a total of 25 cycles.

The products from each PCR reaction (resulting in the NP1 region and the signal region) are then used as template in another PCR reaction: Samples from each reaction are combined in a one-to-one ratio and subjected to the PCR using primers "SIG-L" and "NP1-R" at 94°C for 1 min., 50°C for 2 min., 72°C for 3 min. for a total of 25 cycles. Products of the PCR were then digested with Asp718 and BamHI and cloned into pCGN1431 (see below).

Following digestion with BamHI and Asp718, the thionin signal/NP1 DNA sequence was cloned into BamHI-Asp718 digested pCGN1431. The sequence of the thionin signal/NP1 fusion was confirmed by dideoxy chain termination DNA sequencing (Sanger, et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)). The resulting construct has been designated pCGN3700.

The expression cassette is then cloned into a binary vector, such as pCGN1557 (described below) and the resulting construct is introduced into Agrobacterium tumefaciens by transformation. The transformed A. tumefaciens is then used to generate transformed plants, as described in Example 7.

Construction of pCGN1431

Plasmid pCGN1431 is described in co-pending application U.S. Serial No. 07/225,332, which is incorporated herein by this reference.

Plasmid pCGN1431 contains a double Cauliflower Mosaic Virus (CaMV) promoter and the tml-3' region with multiple cloning sites between them. This promoter/terminator cassette is contained in a pUC-derived vector which contains a chloramphenicol resistance gene. The cassette is bordered by multiple restriction sites for easy removal. The plasmid is derived by a series of steps (see below) from an initial double 35S plasmid, pCGN2113, which itself is derived from pCGN164 and pCGN638. The plasmid pCGN2113 was deposited with the ATCC on March 22, 1989, under accession number 40587.

The SalI-EcoRI fragment of pCGN2113 (see below), which contains the entire promoter-polylinker-3' cassette, is removed by SalI-EcoRI digestion and cloned into SalI-EcoRI digested pCGN565 to create pCGN2120; pCGN565 is cloning vector based on pUC12-Cm (K. Buckley, "Regulation and expression of the ϕX174 lysis gene", Ph.D. Thesis, University of California, San

Diego, 1985), but containing the polylinker from pUC18 (Norrander et al., Gene 26:101-106 (1983)). Plasmid pCGN2120 is digested to completion with PstI and religated. A clone is selected which has deleted only the 858bp PstI-PstI fragment (bp 9207-10065, Barker et al., Plant Mol. Bio. 2:335-350 (1983)) from the tml-3' region to create pCGN1431.

Construction of pCGN2113

Plasmid pCGN164 (see below) is digested with EcoRV and BamHI to release a EcoRV-BamHI fragment which contains a portion of the 35S promoter (bp 7340-7433, Gardner et al., Nucl. Acids Res. 9:2871-2888 (1981)). Plasmid pCGN638 (see below) is digested with HindIII and EcoRV to release a HindIII-EcoRV fragment containing a different portion of the 35S promoter (bp 6493-7340). These two fragments are ligated into pCGN986 (see below) which has been digested with HindIII and BamHI to remove the HindIII-BamHI fragment containing the 35S promoter. This ligation produces pCGN639, which contains the backbone and tml-3' region from pCGN986 and the two 35S promoter fragments from pCGN164 and pCGN638. Plasmid pCGN638 is digested with EcoRV and DdeI to release a fragment of the 35S promoter (bp 7070-7340). The fragment is treated with Klenow fragment to create blunt ends, and is ligated into the EcoRV site of pCGN639 to produce pCGN2113, having the fragment in the proper orientation.

Construction of pCGN164

The AluI fragment of CaMV (bp 7144-7735) (Gardner et al., (1981) supra) is obtained by digestion with AluI and cloned into the HincII site of M13mp7 (Vieira and Messing, Gene 19:259-268 (1982)) to create C614. An EcoRI digest of C614 produces the EcoRI fragment from C614 containing the 35S promoter which is cloned into the EcoRI site of pUC8 (Vieira and Messing, (1982) supra) to produce pCGN146. To trim the promoter

region, the BglII site (bp 7670) is treated with BglII and Bal31 and subsequently a BglII linker is attached to the Bal31 treated DNA to produce pCGN147. Plasmid pCGN147 is digested with EcoRI and HphI and the resultant EcoRI-HphI fragment containing the 35S promoter is ligated into EcoRI-SmaI digested M13mp8 (Vieira and Messing, (1982) supra) to create pCGN164.

Construction of pCGN638

Digestion of CaMV10 (Gardner et al., (1981) supra) with BglII produces a BglII fragment containing a 35S promoter region (bp 6493-7670) which is ligated into the BamHI site of pUC19 (Yanisch-Perron et al., Gene 53:103-119 (1985)) to create pCGN638.

Construction of pCGN1557

Plasmid pCGN1557 (McBride and Summerfelt, Plant Molecular Biology 14(2):269-276 (1990)) is a binary plant transformation vector containing the left and right T-DNA borders of Agrobacterium tumefaciens octopine Ti-plasmid pTiA6 (Currier and Nester, J. Bact. 126:157-165 (1976)), the gentamicin resistance gene of pPh1JI (Hirsch and Beringer, Plasmid 9:2871-2890 (1984)), an Agrobacterium rhizogenes Ri plasmid origin of replication from pLJbB11 (Jouanin et al., Mol. Gen. Genet. 201:370-374 (1985)), a 35S promoter-Kan^r-tml-3′ region capable of conferring kanamycin resistance to transformed plants, a ColE1 origin of replication from pBR322 (Bolivar et al., Gene 2:95-133 (1977)), and a lacZ′ screenable marker gene from pUC18 (Norrander et al., Gene 26:101-106 (1983)).

There are three major intermediate constructs used to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557 backbone, the pRi origin of replication, and the ColE1 origin of replication;

pCGN1546 (see below) contains the CaMV 35S 5′-Kan^r-tml-3′ plant selectable marker region; and

pCGN1541b (see below) contains the right and left T-DNA borders of the A. tumefaciens octopine Ti-plasmid and the lacZ' region from pUC19.

To construct pCGN1557 from the above plasmids, pCGN1546 is digested with XhoI, and the fragment containing the CaMV 35S 5'-Kan^r-tml-3' region is cloned into the XhoI site of pCGN1541b to give the plasmid pCGN1553, which contains T-DNA/left border/CaMV 35S 5'-Kan^r-tml-3'/lacZ'/T-DNA left border. Plasmid pCGN1553 is digested with BglII, and the fragment containing the T-DNA/left border/CaMV 35S 5'-Kan^r-tml-3'/lacZ'/T-DNA left border region is ligated into BamHI digested pCGN1532 to give the complete binary vector, pCGN1557.

Construction of pCGN1532

The 3.5kb EcoRI-PstI fragment containing the gentamicin resistance gene is removed from pPh1JI (Hirsch and Beringer, (1984) supra) by EcoRI-PstI digestion and cloned into EcoRI-PstI digested pUC9 (Vieira and Messing, (1982) supra) to generate pCGN549. HindIII-PstI digestion of pCGN549 yields a 3.1kb fragment bearing the gentamicin resistance gene, which is made blunt ended by Klenow fragment and cloned into PvuII digested pBR322 (Bolivar et al., (1977) supra) to create pBR322Cm. Plasmid pBR322Cm is digested with DraI and SphI, treated with Klenow fragment to create blunt ends, and the 2.8kb fragment cloned into the Ri origin-containing plasmid pLJbB11 (Jouanin et al., (1985) supra) which has been digested with ApaI and made blunt-ended with Klenow fragment, creating pLHbB11Gm. The extra ColE1 origin and the kanamycin resistance gene (Kan^r) are deleted from pLHbB11Gm by digestion with BamHI followed by self closure to create pGmB11. The HindIII site of pGmB11 is deleted by HindIII digestion followed by treatment with Klenow fragment and self closure, creating pGmB11-H. The PstI site of pGmB11-H is deleted by PstI digestion followed

by treatment with Klenow fragment and self closure, creating pCGN1532.

Construction of pCGN1546

The 35S promoter-tml-3′ expression cassette, pCGN986, contains a CaMV 35S promoter and a T-DNA tml-3′-region with multiple restriction sites between them. Plasmid pCGN986 is derived from another cassette, pCGN206, containing a CaMV 35S promoter and a different 3′ region, the CaMV region VI 3′-end. The CaMV 35S promoter is cloned as an AluI fragment (bp 7144-7734) (Gardner et al., (1981) supra) into the HincII site of M13mp7 (Messing et al., Nucl. Acids Res. 9:309-321 (1981)) to create C614. An EcoRI digest of C614 produced the EcoRI fragment from C614 containing the 35S promoter which is cloned into the EcoRI site of pUC8 (Vieira and Messing, (1982) supra) to produce pCGN147.

Plasmid pCGN148a containing a promoter region, selectable marker (Kan^r with 2 ATG's) and 3′ region, is prepared by digesting pCGN528 with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment is cloned into the BglII site of pCGN528 so that the BglII site is proximal to the Kan^r gene of pCGN528.

The shuttle vector used for this construct pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a Kan^r gene (Jorgenson et al., Mol. Gen. Genet. 177:65 (1979)) with HindIII and BamHI and inserting the HindIII-BamHI fragment containing the Kan^r into the HindIII-BamHI site in the tetracycline resistance (Tet^r) gene of pACYC184 (Chang and Cohen, J. Bacteriol. 134:1141-1156 (1978)). Plasmid pCGN526 is made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et al., Cell 19:729-739 (1980)), modified with XhoI linkers inserted into the SmaI site, into the BamHI site of pCGN525. Plasmid pCGN528 is

obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

Plasmid pCGN149a is made by cloning the BamHI-Kan^r fragment from pMB9KanXXI into the BamHI site of pCGN148a. Plasmid pMB9KanXXI is a pUC4K variant (Vieira and Messing, (1982) supra) which has the XhoI site missing, but contains a functional Kan^r gene from Tn903 to allow for efficient selection in Agrobacterium.

Plasmid pCGN149a is digested with HindIII and BamHI and ligated with HindIII and BamHI digested pUC8 to remove the Tn903 Kan^r marker and produce pCGN169. Plasmids pCGN565 (a cloning vector based on pUC12-cm (K. Buckley, (1985) supra) but containing pUC18 linkers) and pCGN169 are both digested with HindIII and PstI and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5′-end of the Tn5 Kan^r gene (up to the PstI site, Jorgenson et al., (1979) supra). A 3′-regulatory region is added to pCGN203 from pCGN204 (an EcoRI fragment of CaMV (bp 408-6105) containing the region VI 3′ cloned into pUC18 (Norrander et al. (1983) supra)) by digestion with HindIII and PstI and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml-3′-sequences are subcloned from the BamHI fragment 19 T-DNA fragment (Thomashow et al., (1980) supra) as a BamHI-EcoRI fragment (bp 9062-12,823, numbering as in Barker et al., (1983) supra) and combined with the pACYC184 (Chang and Cohen, (1978), supra) origin of replication as an EcoRI-HindIII fragment and a gentamicin resistance marker (from plasmid pLB41, obtained from D. Figurski, University of California, San Diego) as a BamHI-HindIII fragment to produce pCGN417.

The unique SmaI site of pCGN417 (nucleotide 11,207 of the Bam19 fragment) is changed to a SacI site using

linkers and the BamHI-SacI fragment is subcloned into pCGN565 to give pCGN971. The BamHI site of pCGN971 is changed to an EcoRI site using linkers. The resulting EcoRI-SacI fragment containing the tml-3′ regulatory sequences is joined to pCGN206 by digestion with EcoRI and SacI to give pCGN975. The small part of the Tn5 Kan^r gene is deleted from the 3′-end of the CaMV 35S promoter by digestion with SalI and BglII, blunting the ends and ligation with SalI linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two SalI sites, an XbaI site, BamHI, SmaI, KpnI and the tml-3′ region (bp 11207-9023 of the T-DNA).

The CaMV 35S promoter-tml-3′ expression cassette, pCGN986 is digested with HindIII. The ends are filled in with Klenow fragment and XhoI linkers are added. The resulting plasmid is called pCGN986X. The BamHI-SacI fragment of pBRX25 (see below) containing the nitrilase gene is inserted into BamHI-SacI digested pCGN986X yielding pBRX66.

Construction of pBRX25 is described in U.S. Patent No. 4,810,648, which is hereby incorporated by reference. Briefly, the method is as follows: The nucleotide sequence of a 1212bp PstI-HincII DNA segment encoding the bromoxynil-specific nitrilase contains 65bp of 5′-untranslated nucleotides. To facilitate removal of a portion of these excess nucleotides, plasmid pBRX9 is digested with PstI, and treated with nuclease Bal31. BamHI linkers are added to the resulting ends. BamHI-HincII fragments containing a functional bromoxynil gene are cloned into the BamHI-SmaI sites of pCGN565. The resulting plasmid, pBRX25, contains only 11bp of 5′-untranslated bacterial sequence.

Plasmid pBRX66 is digested with PstI and EcoRI, blunt ends generated by treatment with Klenow fragment,

and XhoI linkers added. The resulting plasmid pBRX68 now has a tml-3′ region that is approximately 1.1kb. Plasmid pBRX68 is digested with SalI and SacI, blunt ends generated by treatment with Klenow fragment and EcoRI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter-tml-3′ expression cassette lacking the nitrilase gene.

The Tn5 Kan^r gene is then inserted into pCGN986XE. The 1.0kb EcoRI fragment of pCGN1536 (see below) is ligated into pCGN986XE digested with EcoRI. A clone with Kan^r in the correct orientation for transcription and translation is chosen and called pCGN1537b. The 35S promoter Kan^R-tml-3′ region is then transferred to a chloramphenicol resistant (Cam^r) plasmid backbone. Plasmid pCGN786, (a pUC-CAM based vector with the synthetic oligonucleotide
5′ GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3′
containing the cloning sites EcoRI, SalI, BglII, PstI, XhoI, BamHI, and HindIII inserted into pCGN566; pCGN566 contains the EcoRI-HindIII linker of pUC18 inserted into the EcoRI-HindIII sites of pUC13-cm (K. Buckley, (1985) supra)) is digested with XhoI and the XhoI fragment of pCGN1537b containing the 35S promoter Kan^r-tml-3′ region is ligated in. The resulting clone is termed pCGN1546.

Construction of pCGN1536

The 5.4kb EcoRI fragment is removed from pVK232 (Knauf and Nester, Plasmid 8:45 (1982)) by EcoRI digestion and cloned into EcoRI digested pACYC184 (Chang and Cohen, (1978) supra) to create pCGN14. The 1434bp ClaI-SphI fragment of pCGN14, containing the mas-5′ region (bp 20128-21562 according to numbering of Barker et al., (1983) supra) is cloned into AccI-SphI digested pUC19 (Yanisch-Perron et al., (1985) supra) to generate pCGN40. A 746bp EcoRV-NaeI fragment of the mas-5′ region is replaced by an XhoI site by digesting

pCGN40 with EcoRV and NaeI followed by ligation in the presence of a synthetic XhoI linker DNA to create pCGN1036. The 765bp SstI-HindIII fragment (bp 18474-19239) of pCGN14, containing the mas-3′ region, is cloned into SstI-HindIII digested pUC18 (Norrander et al., (1983) supra) to yield pCGN43. The HindIII site of pCGN43 is replaced with an EcoRI site by digestion with HindIII, blunt ending with Klenow fragment, and ligation of synthetic EcoRI linker DNA to create pCGN1034. The 767bp EcoRI fragment of pCGN1034 is cloned into EcoRI-digested pCGN1036 in the orientation that places nucleotide 19239 of the mas-3′ region proximal to the mas-5′ region to create pCGN1040. Plasmid pCGN1040 is subjected to partial digestion with SstI, treated with T4 DNA polymerase to create blunt ends, and ligated in the presence of synthetic XhoI linker DNA. A clone is selected in which only the SstI site at the junction of nucleotide 18474 and vector DNA (constructed in pCGN43 and carried into pCGN1040) is replaced by an XhoI site to generate pCGN1047.

Plasmid pCGN565 (see above) is digested with EcoRI and HindIII, treated with Klenow fragment to create blunt ends, and ligated in the presence of synthetic XhoI linker DNA to create pCGN1003. This recreates the EcoRI site adjacent to the XhoI linker. Plasmid pCGN1003 is digested with EcoRI, treated with Klenow fragment to create blunt ends, and ligated in the presence of synthetic PstI linker DNA to create pCGN1007. The 1.5kb XhoI fragment of pCGN1047, containing the mas-5′ region and the mas-3′ region with multiple cloning sites between, is cloned into XhoI-digested pCGN1007 to construct pCGN1052. A portion of the multiple cloning site of pCGN1052 is deleted by digestion with XbaI and SstI, treated with Klenow fragment to make blunt ends, and ligated to generate pCGN1052ΔX5.

The 1kb EcoRI-SmaI fragment of pCGN783 (pCGN783 is a binary plasmid containing the left and right T-DNA borders of A. tumefaciens (Barker et al., (1983) supra) the gentamicin resistance gene of pPH1JI (Hirsch and Beringer, (1984) supra), the Kan^r gene of Tn5 (Jorgenson et al., (1979) supra and Wolff et al., Nuc. Acids Res. 13:355-367 (1985)) and the 3′ region from transcript 7 of pTiA6 (Barker et al., (1983) supra; plasmid pCGN783 has been deposited with the ATCC in Rockville, MD, on December 23, 1988 under accession number 67868), containing the 1 ATG-Kan^r gene, is cloned into EcoRI-SmaI digested Bluescript M13-KS (Strategene, Inc., San Diego, CA) to create pBSKm; this plasmid contains an M13 region allowing generation of single-stranded DNA. Single-stranded DNA is generated according to the supplier's recommendations, and in vitro mutagenesis is performed (Adelman et al., DNA 2:183-193 (1983)) using a synthetic oligonucleotide with the sequence 5′ GAACTCCAGGACGAGGC 3′ to alter a PstI site with the Kan^r gene and make it undigestible, creating pCGN1534. Plasmid pCGN1534 is digested with SmaI and ligated in the presence of synthetic EcoRI linker DNA to generate pCGN1535.

The 1kb EcoRI fragment of pCGN1535 is cloned into EcoRI-digested pCGN1052ΔX5 to create the mas-5′-Kan^r-mas-3′ plant selectable marker cassette pCGN1536.

Construction of pCGN1541b

Plasmid pCGN565RBα2X (see below) is digested with BglII and XhoI, and the 728bp fragment containing the T-DNA right border piece and the lacZ′ gene is ligated with BglII- and XhoI-digested pCGN65ΔKX-S+K (see below), replacing the BglII-XhoI right border fragment of pCGN65ΔKX-S+K. The resulting plasmid, pCGN65α2X contains both T-DNA borders and the lacZ′ gene. The ClaI fragment of pCGN65α2X is replaced with an XhoI site by digesting with ClaI, blunting the ends using

Klenow fragment, and ligating with XhoI linker DNA, resulting in plasmid pCGN65α2XX. Plasmid pCGN65α2XX is digested with BglII and EcoRV, treated with Klenow fragment to create blunt ends, and ligated in the presence of BglII linker DNA, resulting in pCGN65α2XX'. Plasmid pCGN65α2XX' is digested with BglII and ligated with BglII-digested pCGN1538 (see below), resulting in pCGN1541a, which contains both plasmid backbones. Plasmid pCGN1541a is digested with XhoI and religated. Ampicillin resistant (Amp^r), chloramphenicol sensitive clones are chosen, which lack the pACYC184-derived backbone, creating pCGN1541b.

Plasmid pCGN1538 is generated by digesting pBR322 with EcoRI and PvuII, treating with Klenow fragment to generate blunt ends, and ligating with BglII linkers. Plasmid pCGN1538 is ampicillin resistant, tetracycline sensitive.

Construction of pCGN65ΔKX-S+K

Plasmid pCGN501 is constructed by cloning a 1.85kb EcoRI-XhoI fragment of pTiA6 (Currier and Nester, (1976) supra) containing bases 13362-15208 (Barker et al., (1983) supra) of the T-DNA (right border), into EcoRI- and SalI-digested M13mp9 (Vieira and Messing, (1982) supra). Plasmid pCGN502 is constructed by cloning a 1.6kb HindIII-SmaI fragment of pTiA6, containing bases 602-2212 of the T-DNA (left border), into HindIII- and SmaI-digested M13mp9. Plasmids pCGN501 and pCGN502 are both digested with EcoRI and HindIII and both T-DNA-containing fragments cloned together into HindIII digested pUC9 (Vieira and Messing, (1982) supra) to yield pCGN503, containing both T-DNA border fragments. Plasmid pCGN503 is digested with HindIII and EcoRI and the two resulting HindIII-EcoRI fragments (containing the T-DNA borders) are cloned into EcoRI-digested pHC79 (Hohn and Collins, Gene 11:291-298 (1980)) to generate pCGN518. The 1.6kb

KpnI-EcoRI fragment from pCGN518, containing the left T-DNA border, is cloned into KpnI- and EcoRI-digested pCGN565 to generate pCGN580. The BamHI-BglII fragment of pCGN580 is cloned into the BamHI site of pACYC184 (Chang and Cohen, (1978) supra) to create pCGN51. The 1.4kb BamHI-SphI fragment of pCGN60, containing the T-DNA right border fragment, is cloned into BamHI- and SphI-digested pCGN51 to create pCGN65, which contains the right and left T-DNA borders.

Plasmid pCGN65 is digested with KpnI and XbaI, treated with Klenow fragment to create blunt ends, and ligated in the presence of synthetic BglII linker DNA to create pCGN65ΔKX. Plasmid pCGN65ΔKX is digested with SalI, treated with Klenow fragment to create blunt ends, and ligated in the presence of synthetic XhoI linker DNA to create pCGN65ΔKX-S+X.

Construction of pCGN565RBα2X

Plasmid pCGN451 (see below) is digested with HpaI and ligated in the presence of synthetic SphI linker DNA to generate pCGN55. The XhoI-SphI fragment of pCGN55 (bp 13800-15208, including the right border of T-DNA (Barker et al., Gene 2:95-113 (1977)) is cloned into SalI- and SphI-digested pUC19 (Yanisch-Perron et al., (1985) supra) to create pCGN60. The 1.4kb HindIII-BamHI fragment of pCGN60 is cloned into HindIII- and BamHI-digested pSP64 (Promega, Inc.) to generate pCGN1039. Plasmid pCGN1039 is digested with SmaI and NruI (deleting bp 14273-15208; (Barker et al., (1977) supra)) and ligated in the presence of synthetic BglII linker DNA creating pCGN1039ΔNS. The 0.47kb EcoRI-HindIII fragment of pCGN1039ΔNS is cloned into EcoRI- and HindIII-digested pCGN565 to create pCGN565RB. The HindIII site of pCGN565RB is replaced with an XhoI site by digesting with HindIII, treating with Klenow fragment, and ligating in the presence of synthetic XhoI linker DNA to create pCGN565RB-H+X.

Plasmid pUC18 (Norrander et al., (1983) supra) is digested with HaeII to release the lacZ' fragment, treated with Klenow fragment to create blunt ends, and the lacZ'-containing fragment is ligated into pCGN565RB-H+X, which had been digested with AccI and SphI and treated with Klenow fragment, in such an orientation that the lacZ' promoter is proximal to the right border fragment; this construct, pCGN565RBα2x is positive for lacZ' expression when plated on an appropriate host and contains bp 13990-14273 of the right border fragment (Barker et al., (1977) supra) having deleted the AccI-SphI fragment (bp 13800-13990).

Construction of pCGN451

Plasmid pCGN451 contains an ocs5'-ocs3' cassette, including the T-DNA right border, cloned into a derivative of pUC8 (Vieira and Messing, (1982) supra). The modified vector is derived by digesting pUC8 with HincII and ligating in the presence of synthetic linker DNA, creating pCGN416, and then deleting the EcoRI site of pCGN416 by EcoRI digestion followed by treatment with Klenow fragment and self-ligation to create pCGN426.

The ocs5'-ocs3' cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid pTiA6 (Currier and Nester, (1976) supra). To generate the 5'end, which includes the T-DNA right border, an EcoRI fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker et al., (1977) supra for the closely related Ti plasmid pTi15955)) is removed from pVK232 (Knauf and Nester, (1982) supra) by EcoRI digestion and cloned into EcoRI digested pACYC184 (Chang and Cohen, (1978) supra) to generate pCGN15.

The 2.4kb BamHI-EcoRI fragment (bp 13774-16202) of pCGN15 is cloned into EcoRI- and BamHI-digested pBR322 (Bolivar et al., (1977) supra) to yield pCGN429. The 412bp EcoRI-BamHI fragment (bp 13362-13772) of pCGN15

is cloned into EcoRI- and BamHI-digested pBR322 to yield pCGN407. The cut-down promoter fragment is obtained by digesting pCGN407 with XmnI (bp 13512), followed by resection with Bal31 exonuclease, ligation of synthetic EcoRI linkers, and digestion with BamHI. Resulting fragments of approximately 130bp are gel purified and cloned into M13mp9 (Vieira and Messing, (1982) supra) and sequenced. A clone, I-4, in which the EcoRI linker has been inserted at position 13642 between the transcription initiation point and the translation initiation codon, is identified by comparison with the sequence of de Greve et al., J. Mol. Appl. Genet. 1:499-512 (1982). The EcoRI cleavage site is at position 13639, downstream from the mRNA start site. The 141bp EcoRI-BamHI fragment of I-4, containing the cut-down promoter, is cloned into EcoRI- and BamHI-digested pBR322 to create pCGN428. The 141bp EcoRI-BamHI promoter piece from pCGN428 and the 2.5kb EcoRI-BamHI ocs5′ piece from pCGN429 are cloned together into EcoRI-digested pUC19 (Vieira and Messing, (1982) supra) to generate pCGN442, reconstructing the ocs upstream region with a cut-down promoter section.

To generate the ocs3′ end, the HindIII fragment of pLB41 (D. Figurski, University of California, San Diego) containing the gentamicin resistance gene is cloned into HindIII-digested pACYC184 (Chang and Cohen, (1978) supra) to create pCGN413b. The 4.7kb BamHI fragment of pTiA6 (Currier and Nester, (1976) supra), containing the ocs3′ region, is cloned into BamHI digested pBR325 (Bolivar, Gene 4:121-136 (1978)) to create 33c-19. The SmaI site at position 11207 (Barker et al., (1977) supra) of 33c-19 is converted to an XhoI site using a synthetic XhoI linker, generating pCGN401.2. The 3.8kb BamHI-EcoRI fragment of pCGN401.2 is cloned into BamHI- and EcoRI-digested pCGN413b to create pCGN419.

The ocs5′-ocs3′ cassette is generated by cloning the 2.64kb EcoRI fragment of pCGN442, containing the 5′ region, into EcoRI-digested pCGN419 to create pCGN446. The 3.1kb XhoI fragment of pCGN446, having the ocs5′ region (bp 13639-15208) and ocs3′ region (bp 11207-12823), is cloned into the XhoI site of pCGN426 to create pCGN451.

Example 6: Synthesis of the Thionin Signal Peptide/Magainin-1 DNA Sequence

The DNA sequence for expression and transport of the AMP magainin M1 is assembled from oligonucleotides using the Klenow fragment of DNA polymerase and the polymerase chain reaction (PCR) technique, as described in U.S. Patent Nos. 4,683,195 and 4,683,202.

The DNA sequence for expression and transport of M1 consists of six non-specific base pairs followed by a BamHI site, then the DNA sequence encoding the putative signal peptide from a barley leaf-specific thionin (to allow for transport out of the cell) fused in-frame to the mature magainin M1 coding region, an Asp718 site, and six more non-specific base pairs. The BamHI and Asp718 sites allow the gene to be cloned into the double 35S expression cassette pCGN1431 (described above); the non-specific bases on the ends are employed to provide double-stranded helix structure on both sides of the restriction sites, which most restriction enzymes require for activity. The codons are selected in the design of this DNA sequence based on codon preferences for dicotyledonous plants (Murray et al., (1989) supra) to allow maximal translation rate in the target plants of the present example (Brassica sp.). Codon selection for other target plant species will be made as a matter of ordinary skill.

The DNA sequence which encodes the putative signal peptide of barley leaf thionin is 127bp in length. Two

overlapping oligonucleotides were designed which, when annealed and extended with Klenow fragment, comprise the entire sequence: "SIG-T" is 73 "sense" strand bases, consisting of bases of 1-73 of the DNA sequence encoding the thionin signal peptide (as described by Gausing, (1987) supra):

"SIG-T":

5′-CATATCTCATTCTACTTGAGAAATTAAGGCCAACCA GCCAACCATGGCAACCAACAAGTCTATTAAGTCCGTG-3′

"SIG-B" is 74 "anti-sense" strand bases, consisting of bases 127-54 of the DNA sequence encoding the signal peptide (Gausing, (1987) supra):

"SIG-B":

5′-GGCTTCAACTTGGACCTGCTCAAGAACCAAACCCAA TATAAGAACACAAATAACCACGGACTTAATAGACTTGT-3′

Bases 54-73 of "SIG-T" and "SIG-B" overlap as complementary strands, and when allowed to anneal they can be extended with Klenow fragment to yield a full length double-stranded DNA fragment of 127bp.

In practice, only a relatively small fraction of the polymerase reaction products will comprise the full 127 base pairs, since the synthesis of long oligomers (>40 bases) is not highly efficient. By employing the PCR as described below, the full length molecules can be selectively amplified, reducing the quantity of short fragments to an insignificant level.

The M1 coding region is 72bp in length. It was constructed in a manner similar to the signal peptide DNA sequence, with "MAG-T" being "sense" bases 1-45, and "MAG-B" being "antisense" bases 72-28, based on magainin M1 DNA sequence as reported by Zasloff (Proc. Natl. Acad. Sci. 84:5449-5453 (1987)).

"MAG-T":
5'-GGAATTGGTAAGTTCTTGCACTCTGCTGGTAAGTTCGGTAAGGCT-3'

"MAG-B":
5'-TTAAGACTTCATAATCTCTCCAACAAAAGCCTTACCGAACTTACC-3'

These oligonucleotides were annealed and repaired with the Klenow fragment as described above.

Four smaller oligonucleotides were synthesized in order to allow fusion of the signal sequence and the M1 sequence, facilitate addition of the restriction sites, and allow for amplification of full-length fragments from the above polymerase reactions:

"SIG-L" is a 33 base oligonucleotide consisting of six non-specific bases, six bases comprising the BamHI site, and the first 21 "sense" bases (1-21) of the signal peptide DNA sequence:
5'-CGAGTTGGATCCVCATATCTCATTCTACTTGAGA-3'

"MAG-R" is a 33 base oligonucleotide consisting of six non-specific bases, six bases comprising the AspI site, and the last 12 "anti-sense" bases (199-178) of the M1 sequence:
5'-TGAGTTGGTACCTTAAGACTTCATAATCTCTCC-3'

"F-SM-T" is a 42 base oligonucleotide consisting of the last 21 "sense" bases of the signal peptide and the first 21 "sense" bases of M1:
5'-GAGCAGGTCCAAGTTGAAGCCGGAATTGGTAAGTTCTTGCAC-3'

"F-SM-B" is a 42 base pair of oligonucleotide complementary to "F-SM-T" consisting of the first 21 "anti-sense" bases of M1 and the last 21 "anti-sense" bases of the signal peptide:
5'-GTGCAAGAACTTACCAATTCCGGCTTCAACTTGGACCTGCTC-3'

When "SIG-L" and "F-SM-B" are used as primers in the PCR with the repaired signal peptide DNA fragment as template, the resulting product comprises six non-specific base pairs and the BamHI site, the signal peptide DNA sequence, and 21 base pairs of the M1 DNA sequence. PCR is performed using a DNA Thermal Cycler machine (Perkin Elmer Cetus; Emeryville, CA) as described above.

When "MAG-R" and "F-SM-T" are used as PCR primers with the repaired M1 DNA fragment as template, the resulting product comprises the 21 base pairs of the signal peptide DNA sequence, the M1 DNA sequence, the Asp718 site, and six non-specific base pairs. Each of these reactions selectively amplifies only the full-length or near full-length fragments from the initial repair reactions, permitting the use of crude oligonucleotides in all steps, and resulting in a great savings of time and manpower.

These two double-stranded products share a 42bp overlap which defines the fusion junction for the thionin signal sequence and the M1 sequence. Upon strand melting (such as occurs during the PCR), a portion of each type will reanneal with an overlapping strand of the other, forming a complex which can repair into the full-length DNA sequence. When the two fragments are mixed in a 1:1 ratio, and amplified in the PCR using "SIG-L" and "MAG-A" as primers, the resulting product is the full-length sequence with the appropriate restriction sites attached.

The actual assembly of the DNA signal/magainin sequence is carried out in several steps. One nmol each of "MAG-T" and "MAG-B" (for the M1 region; "SIG-T" and "SIG-B" are used for the signal region) are combined with 1 μl 100XTE in a total volume of 100μl. This mixture is placed in a DNA Thermal Cycler (Perkin Elmer Cetus; Emeryville, CA) and the DNA strands are

melted and annealed for 30 minutes in a cooling gradient from 80°C to 37°C followed by 15 minutes at 37°C. Ten μl of this reaction is treated with Klenow fragment in a 50μl reaction volume to repair the strands. One μl of the Klenow fragment reaction is used in a PCR reaction with "F-SM-T" and "MAG-A" ("F-SM-B" and "SIG-L") as primers to add the fusion region. The PCR conditions are as follows: 94°C for 1 min., 55°C for 2 min., 72°C for 3 min., for a total of 25 cycles.

The products from each PCR reaction (resulting in the M1 region and the signal region) are then used as template in another PCR reaction: Samples from each reaction are combined in a one-to-one ratio and subjected to PCR using primers "SIG-L" and "MAG-R" at 94°C for 1 min., 50°C for 2 min., 72°C for 3 min., for a total of 25 cycles. PCR products are then digested with Asp718 and BamHI and cloned into pCGN1431.

Following digestion with BamHI and Asp718, the thionin signal/M1 DNA sequence may be cloned into BamHI- and Asp718-digested pCGN1431. The expression cassette is then cloned into a binary vector, such as PCGN1557 (described above), and the resulting construct is introduced into Agrobacterium tumefaciens by transformation. The transformed A. tumefaciens is then used to generate transformed plants, as described in Example 7.

Example 7: Brassica Transformation

Seeds of Brassica napus cv. Westar are soaked in 95% ethanol for 2 min., surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween®20 for 45 min., and rinsed three times in sterile, distilled water.

Treated seeds are then plated in Magenta boxes (Magenta Corp., Chicago, Illinois) with 1/10th

concentration of Murashige minimal organics medium (Gibco) supplemented with pyridoxine (50μg/l), nicotinic acid (50μg/l), glycine (200μg/l), and 0.6% Phytagar (Gibco) at pH 5.8. The seeds are then germinated in a culture room at 22°C in a 16 hour photoperiod with cool fluorescent and red light of intensity approximately 65μ Einsteins per square meter per second ($\mu Em^{-2}S^{-1}$)

Hypocotyls are excised from the resultant 6 day old seedlings, cut into pieces approximately 4mm in length, and plated on feeder plates (Horsch, et al., Science 227:1224-1231 (1985)).

Feeder plates are prepared one day before use by plating 0.5ml of a tobacco suspension culture onto a petri plate (100x25mm) containing about 30ml MS salt base (Carolina Biological), 100mg/l inositol, 1.3mg/l thiamine-HCl, 200mg KH_2PO_4 with 3% sucrose, 1.0mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (termed MS0/1/0 medium). A sterile filter paper disc (Whatman® 3mm) is placed on top of the feeder layer prior to use.

Tobacco suspension cultures are subcultured weekly by transfer of 10ml of culture into 100ml fresh MS medium as described for the feeder plates, but with 0.2mg/l 2,4-D and 0.1mg/l Kinetin.

In embodiments where feeder cells are not used, hypocotyl explants are cut and placed onto a filter paper disc on top of MS0/1/0 medium. All hypocotyl explants are preincubated on feeder plates for 24 hours at 22°C in continuous light of intensity in the range of $30\mu Em^{-2}S^{-1}$ to $65\mu Em^{-2}S^{-1}$.

Single colonies of A. tumefaciens strain EHA 101, containing a binary plasmid, are transferred to 5ml MG/L broth (consisting of 5g mannitol, 1g L-glutamic acid or 1.15g sodium glutamate, 0.5g KH_2PO_4, 0.10g NaCl, 0.10g $MgSO_4 \cdot 7H_2O$, 1μg biotin, 5g tryptone, 2.5g yeast

extract (per liter, pH adjusted to 7.0)), and grown overnight at 30°C.

Hypocotyl explants are then immersed in 7-12ml MG/L broth with bacteria diluted to $1x10^8$ bacteria/ml and after 10-25 min. are transferred onto feeder plates. After 48 hours of co-incubation with Agrobacterium, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium (B5 salts and vitamins supplemented with 1mg/l 2,4-D and 3% sucrose - Gamborg, et al., Exp. Cell Res. 50:151-158 (1968)) which contains filter sterilized carbenicillin (500mg/l, added after autoclaving) and 25mg/l kanamycin sulfate (Boehringer Mannheim).

After 3 days in culture at 65-75 $\mu Em^{-2}S^{-1}$ continuous light, hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3mg/l benzylaminopurine, 1mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500mg/l) and kanamycin sulfate (25mg/l). Hypocotyl explants were subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, 300mg/l carbenicillin, 50mg/l kanamycin sulfate and 0.6% Phytagar. After 2-4 weeks, shoots which remain green were cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2mg/l indolebutyric acid, 50mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPTII activity.

In accordance with one aspect of the subject

invention, plant-associated pathogen resistance can be imparted to susceptible hosts to provide for enhanced protection of the host crop. In addition, DNA sequences are provided which can be used for producing selected AMPs, for example, in methods of the present invention. Furthermore, a method is provided for inhibiting plant associated pathogens by applying selected AMPs. Thus, plants can be grown which will be less susceptible to a variety of plant-associated pathogens in order to greatly enhance the utility of the crop plants and allow their growth in regions wherein plant associated pathogen infestation is endemic.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teaching of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Claims

1. A method of limiting growth of a plant-associated pathogen comprising contacting a plant-associated pathogen with a plant-associated pathogen-inhibiting amount of an animal-derived anti-microbial peptide under growth limiting conditions and allowing said anti-microbial peptide to act upon said plant-associated pathogen thereby limiting growth by said plant-associated pathogen.

2. The method of Claim 1 wherein said animal-derived antimicrobial peptide is selected from the family of magainins.

3. The method of Claim 1 wherein said animal-derived antimicrobial peptide is selected from the family of defensins.

4. The method of Claim 1 wherein said plant associated pathogen is a fungus.

5. The method of Claim 4 wherein said fungus is selected from the group consisting of Plasmodiophormycetes species, Chytridiomycetes species, Zygomycetes species, Oomycetes species, Ascomycetes species, Basidiomycetes species and Deuteromycetes species.

6. The method of Claim 5 wherein said fungus is selected from the group consisting of Fusarium oxysporum, Pythium ultimum, and Verticillium albo-atrium.

7. The method of Claim 1 wherein said plant associated pathogen is a bacterium.

8. The method of Claim 6 wherein said bacterium is selected from the group consisting of Agrobacterium species, Corynebacterium species, Erwinia species, Pseudomonas species, Xanthomonas species, Streptomyces species and Rhizobium species.

9. The method of Claim 7 wherein said bacterium is selected from the group consisting of Erwinia carotovora and Pseudomonas syringae, and Xanthamonas campestris.

10. The method of Claim 1 wherein said growth is inhibited.

11. The method of Claim 1 wherein said growth is substantially slowed.

12. A DNA construct comprising a transcriptional initiation region functional in plants joined at its 3' terminus to the 5' terminus of a DNA sequence which codes for at least a portion of a signal peptide which is secreted from a plant cell and an animal-derived anti-microbial peptide.

13. The DNA construct of Claim 12 wherein said animal-derived antimicrobial peptide is selected from the family of magainins.

14. The DNA construct of Claim 13 wherein said DNA sequence codes for a peptide selected from the group consisting of:

gly-ile-gly-lys-phe-leu-his-ser-ala-gly-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-lys-ser,

gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser, and

gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn.

15. The DNA construct of Claim 12 wherein said animal-derived antimicrobial peptide is selected from the family of defensins.

16. The DNA construct of Claim 15 wherein said DNA sequence codes for a peptide selected from the group consisting of:

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg,

gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg,

val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg, and

val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-asn-gly-val-arg-his-thr-leu-cys-cys-arg.

17. The DNA construct of Claim 12 wherein said plant associated pathogen is a fungus.

18. The DNA construct of Claim 17 wherein said fungus is selected from the group consisting of Plasmodiophormycetes species, Chytridiomycetes species, Zygomycetes species, Oomycetes species, Ascomycetes species, Basidiomycetes species, and Deuteromycetes species.

19. The DNA construct of Claim 18 wherein said fungus is selected from the group consisting of Fusarium oxysporum, Pythium ultimum, and Verticillium albo-atrium.

20. The DNA construct of Claim 12 wherein said plant associated pathogen is a bacterium.

21. The DNA construct of Claim 20 wherein said bacterium is selected from the group consisting of Agrobacterium species, Corynebacterium species, Erwinia species, Pseudomonas species, Xanthomonas species, Streptomyces species and Rhizobium species.

22. The DNA construct of Claim 21 wherein said bacterium is selected from the group consisting of Erwinia carotovora and Pseudomonas syringae, and Xanthamonas campestris.

23. A method for improving disease resistance in plants which comprises:

modifying a plant with a DNA construct comprising a transcriptional initiation region functional in plants joined at its 3' terminus to the 5' terminus of a DNA sequence which codes for a structural gene for at least a portion of a

signal peptide which is secreted from a plant cell and an animal-derived anti-microbial peptide, said structural gene being under the transcriptional control of said plant host and being capable, in said plant host, of producing a plant-associated pathogen-inhibiting amount of said anti-microbial peptide under growth limiting conditions.

24. The method of Claim 23 wherein said animal-derived antimicrobial peptide is selected from the family of magainins.

25. The method of Claim 24 wherein said structural gene codes for a peptide selected from the group consisting of:

gly-ile-gly-lys-phe-leu-his-ser-ala-gly-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-lys-ser,

gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser, and

gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn.

26. The DNA construct of Claim 23 wherein said animal-derived antimicrobial peptide is selected from the family of defensins.

27. The method of Claim 26 wherein said structural gene codes for a peptide selected from the group consisting of:

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg,

gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg,

val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg, and

val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-asn-gly-val-arg-his-thr-leu-cys-cys-arg.

28. The method of Claim 23 wherein said plant associated pathogen is a fungus.

29. The method of Claim 28 wherein said fungus is selected from the group consisting of Plasmodiophormycetes species, Chytridiomycetes species, Zygomycetes species, Oomycetes species, Ascomycetes species, Basidiomycetes species and Deuteromycetes species.

30. The method of Claim 29 wherein said fungus is selected from the group consisting of Fusarium oxysporum, Pythium ultimum, and Verticillium albo-atrium.

31. The method of Claim 23 wherein said plant associated pathogen is a bacterium.

32. The method of Claim 31 wherein said bacterium is selected from the group consisting of Agrobacterium species, Corynebacterium species, Erwinia species, Pseudomonas species, Xanthomonas species, Streptomyces species and Rhizobium species.

33. The method of Claim 32 wherein said bacterium is selected from the group consisting of Erwinia carotovora and Pseudomonas syringae, and Xanthamonas campestris.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01969

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02; C07K 7/10; C12N 15/12

U.S. CL.: 435/172.1; 514/12, 13; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/172.1; 514/12,13 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US, A, 4,543,252 (LEHRER ET AL.) 24 September 1985 See entire document.	1-11
X	US, A, 4,659,692 (LEHRER ET AL.) 21 April 1987 See entire document.	1-11
X	US, A, 4,810,777 (ZASLOFF) 07 March 1989 See entire document.	1-11
Y	BioEssays, "Increasing Bacterial Disease Resistance In Plants Utilizing Antibacterial Genes From Insects" Volume 6, No. 6, pp 263-270, (JAYNES ET AL.) June 1987, See entire document.	12-33
Y,P	Science, "An Assay For Circulating Antibodies To A Major Etiologic Virus Of Human Non-A, Non-B Hepatitis", Vol. 244, pp362-364, (KUO ET AL.) 21 April 1989.	12-33

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
02 JULY 1990	02 AUG 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	LESTER L. LEE

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Science, "Inheretance of Functional Foreign Genes in Plants", Vol. 223, pp 496-498, (HORSCH ET AL.), January 1984, pp 496-498, See entire document.	12-33
Y,P	Science, "Isolation of a cDNA Clone Derived From a Blood-Borne Non-A, Non-B Viral Hepatitis Genome", Vol. 244, pp 359-362, (CHOO ET AL.) 21 April 1989.	12-33

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.